ATENT COOPERATION TRE. TY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and Administrative Instructions, Section 422)

To:

MINOJA, Fabrizio
Bianchetti Bracco Minoja S.r.l.
Via Rossini, 8
I-20122 Milano
ITALIE

Date of mailing (day/month/year)
24 February 2000 (24.02.00)

Applicant's or agent's file reference
SCB451PCT

IMPORTANT NOTIFICATION

International application No.
PCT/IT98/00231

International filing date (day/month/year)
11 August 1998 (11.08.98)

1. *The following indications appeared on record concerning:*

[X] the applicant [ ] the inventor [ ] the agent [ ] the common representative

Name and Address

State of Nationality | State of Residence

Telephone No.

Facsimile No.

Teleprinter No.

2. *The International Bureau hereby notifies the applicant that the following change has been recorded concerning:*

[ ] the person [ ] the name [ ] the address [ ] the nationality [ ] the residence

Name and Address

EUROPEAN COMMUNITY, represented by
THE COMMISSION OF THE EUROPEAN COMMUNITIES
rue Alcide De Gasperi
L-2920 Luxembourg
Luxembourg

State of Nationality: LU | State of Residence: LU

Telephone No.

Facsimile No.

Teleprinter No.

3. Further observations, if necessary:
Addition of applicant for all designated States except US. Power of attorney authorizing MINOJA, Fabrizio to represent the applicant EUROPEAN COMMUNITY represented by THE COMMISSION OF THE EUROPEAN COMMUNITIES is required.

4. A copy of this notification has been sent to:

[X] the receiving Office
[ ] the International Searching Authority
[X] the International Preliminary Examining Authority
[ ] the designated Offices concerned
[X] the elected Offices concerned
[ ] other:

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer
Mougamadou ABIDINE

Telephone No.: (41-22) 338.83.38

ATENT COOPERATION TRE. .Y

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and Administrative Instructions, Section 422)

To:

MINOJA, Fabrizio
Bianchetti Bracco Minoja S.r.l.
Via Rossini, 8
I-20122 Milano
ITALIE

Date of mailing (day/month/year)
24 February 2000 (24.02.00)

Applicant's or agent's file reference
SCB451PCT

IMPORTANT NOTIFICATION

International application No.
PCT/IT98/00231

International filing date (day/month/year)
11 August 1998 (11.08.98)

1. The following indications appeared on record concerning:
[X] the applicant [X] the inventor [ ] the agent [ ] the common representative

Name and Address
RONCUCCI, Romeo
(Deceased)

State of Nationality: IT
State of Residence: IT
Telephone No.:
Facsimile No.:
Teleprinter No.:

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:
[ ] the person [ ] the name [ ] the address [ ] the nationality [ ] the residence

Name and Address
DELACHET, Anne, Georgette, Christiane
legal representative of RONCUCCI, Roxanne
(Hieress of RONCUCCI, Romeo (deceased)
Avenue Brancolard 119/A
Nice
France

State of Nationality: IT
State of Residence: FR
Telephone No.:
Facsimile No.:
Teleprinter No.:

3. Further observations, if necessary:
The person indicated in Box 2 is for the purposes of United States of America only.

4. A copy of this notification has been sent to:
[X] the receiving Office
[ ] the International Searching Authority
[X] the International Preliminary Examining Authority
[ ] the designated Offices concerned
[X] the elected Offices concerned
[ ] other:

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland
Facsimile No.: (41-22) 740.14.35

Authorized officer
Mougamadou ABIDINE
Telephone No.: (41-22) 338.83.38

Copy for th Elected Offic (EO/US) PCT/IT98/00231

ATENT COOPERATION TRE. .Y

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and Administrative Instructions, Section 422)

To:

MINOJA, Fabrizio
Bianchetti Bracco Minoja S.r.l.
Via Rossini, 8
I-20122 Milano
ITALIE

Date of mailing (day/month/year) 24 February 2000 (24.02.00)	
Applicant's or agent's file reference SCB451PCT	**IMPORTANT NOTIFICATION**
International application No. PCT/IT98/00231	**International filing date** (day/month/year) 11 August 1998 (11.08.98)

1. The following indications appeared on record concerning:

[X] the applicant [ ] the inventor [ ] the agent [ ] the common representative

Name and Address	State of Nationality	State of Residence
RONCUCCI, Romeo (Deceased)	IT	IT
	Telephone No.	
	Facsimile No.	
	Teleprinter No.	

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

[ ] the person [ ] the name [ ] the address [ ] the nationality [ ] the residence

Name and Address	State of Nationality	State of Residence
CASTAGNOLI, Maria Novella, legal representative of RONCUCCI, Rachele (Hieress of RONCUCCI, Romeo (deceased)) via Ungaretti 17 I-20028 San Vittore Olona Italy	IT	IT
	Telephone No.	
	Facsimile No.	
	Teleprinter No.	

3. Further observations, if necessary:
The person indicated in Box 2 is for the purposes of United States of America only.

4. A copy of this notification has been sent to:

[X] the receiving Office
[ ] the International Searching Authority
[X] the International Preliminary Examining Authority
[ ] the designated Offices concerned
[X] the elected Offices concerned
[ ] other:

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer Mougamadou ABIDINE
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

Form PCT/IB/306 (March 1994) 003130342

Copy for the Elected Office (EO/US) PCT/IT98/00231

ATENT COOPERATION TRE. .TY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and Administrative Instructions, Section 422)

To:

MINOJA, Fabrizio
Bianchetti Bracco Minoja S.r.l.
Via Rossini, 8
I-20122 Milano
ITALIE

Date of mailing (day/month/year)
24 February 2000 (24.02.00)

Applicant's or agent's file reference
SCB451PCT

IMPORTANT NOTIFICATION

International application No.
PCT/IT98/00231

International filing date (day/month/year)
11 August 1998 (11.08.98)

1. The following indications appeared on record concerning:

[X] the applicant [ ] the inventor [ ] the agent [ ] the common representative

Name and Address

State of Nationality

State of Residence

Telephone No.

Facsimile No.

Teleprinter No.

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

[ ] the person [ ] the name [ ] the address [ ] the nationality [ ] the residence

Name and Address
GENE CONTROL S.A.
9, rue Boissonnas
CH-1211 Geneve
Switzerland

State of Nationality: CH

State of Residence: CH

Telephone No.

Facsimile No.

Teleprinter No.

3. Further observations, if necessary:
Addition of applicant for all designated States except US. Power of attorney authorizing MINOJA, Fabrizio to represent the applicant GENE CONTROL S.A. is required.

4. A copy of this notification has been sent to:

[X] the receiving Office
[ ] the International Searching Authority
[X] the International Preliminary Examining Authority
[ ] the designated Offices concerned
[X] the elected Offices concerned
[ ] other:

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer
Mougamadou ABIDINE

Telephone No.: (41-22) 338.83.38

Copy for the Elected Office (EO/US) PCT/IT98/00231

ATENT COOPERATION TRE TY

From the INTERNATIONAL BUREAU

To:

MINOJA, Fabrizio
Bianchetti Bracco Minoja S.r.l.
Via Rossini, 8
I-20122 Milano
ITALIE

PCT

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and Administrative Instructions, Section 422)

Date of mailing (day/month/year) 24 February 2000 (24.02.00)	
Applicant's or agent's file reference SCB451PCT	**IMPORTANT NOTIFICATION**
International application No. PCT/IT98/00231	**International filing date** (day/month/year) 11 August 1998 (11.08.98)

1. The following indications appeared on record concerning:

[X] the applicant [ ] the inventor [ ] the agent [ ] the common representative

Name and Address	State of Nationality	State of Residence
RONCUCCI, Romeo (Deceased)	IT	IT
	Telephone No.	
	Facsimile No.	
	Teleprinter No.	

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

[ ] the person [ ] the name [ ] the address [ ] the nationality [ ] the residence

Name and Address	State of Nationality	State of Residence
RONCUCCI, Sylvie via Thaon di Revel, 12 I-20159 Milano Italy (Hieress of RONCUCCI, Romeo (deceased))	IT	IT
	Telephone No.	
	Facsimile No.	
	Teleprinter No.	

3. Further observations, if necessary:
The person indicated in Box 2 is for the purposes of United States of America only.

4. A copy of this notification has been sent to:

[X] the receiving Office
[ ] the International Searching Authority
[X] the International Preliminary Examining Authority
[ ] the designated Offices concerned
[X] the elected Offices concerned
[ ] other:

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer Mougamadou ABIDINE
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

Form PCT/IB/306 (March 1994) 003130334

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

United States Patent and Trademark Office
(Box PCT)
Crystal Plaza 2
Washington, DC 20231
ÉTATS-UNIS D'AMÉRIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 03 May 1999 (03.05.99)	
International application No. PCT/IT98/00231	**Applicant's or agent's file reference** SCB451PCT
International filing date (day/month/year) 11 August 1998 (11.08.98)	**Priority date** (day/month/year) 28 August 1997 (28.08.97)
Applicant SACCO, Maria, Grazia et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

25 March 1999 (25.03.99)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer S. Mafla
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

PCT

REQUEST

The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty.

For receiving Office use only

International Application No.

International Filing Date

Name of receiving Office and "PCT International Application"

Applicant's or agent's file reference *(if desired) (12 characters maximum)* SCB451PCT

Box No. I TITLE OF INVENTION ANIMALI TRANSGENICI PER LO STUDIO DI AGENTI TOSSICI CHIMICI, FISICI O BIOLOGICI

Box No. II APPLICANT

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

CONSIGLIO NAZIONALE DELLE RICERCHE
Piazzale Aldo Moro, 7
00185 ROMA
Italy

☐ This person is also inventor.

Telephone No.

Facsimile No.

Teleprinter No.

State *(that is, country)* of nationality: ITALY

State *(that is, country)* of residence: ITALY

This person is applicant for the purposes of: ☐ all designated States ☒ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box

Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

SACCO, Maria Grazia
Institute of Advanced Biomedical Technologies
CONSIGLIO NAZIONALE DELLE RICERCHE
Via Ampere, 56
20131 MILANO Italy

This person is:

☐ applicant only

☒ applicant and inventor

☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality: ITALY

State *(that is, country)* of residence: ITALY

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

☒ Further applicants and/or (further) inventors are indicated on a continuation sheet.

Box No. IV AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE

The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as: ☒ agent ☐ common representative

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

MINOJA, Fabrizio
BIANCHETTI BRACCO MINOJA S.r.l.
Via Rossini, 8
20122 MILANO
Italy

Telephone No. 0039.02.76021218

Facsimile No. 0039.02.783078

Teleprinter No.

☐ **Address for correspondence:** Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.

Continuation of Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)

If none of the following sub-boxes is used, this sheet should not be included in the request.

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

ZECCA, Luigi
Institute of Advanced Biomedical Technologies
CONSIGLIO NAZIONALE DELLE RICERCHE
Via Ampere, 56
20131 MILANO Italy

This person is:
- [ ] applicant only
- [X] applicant and inventor
- [ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality: ITALY

State *(that is, country)* of residence: ITALY

This person is applicant for the purposes of:
- [ ] all designated States
- [ ] all designated States except the United States of America
- [X] the United States of America only
- [ ] the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

BROMLEY, Peter
Institute of Advanced Biomedical Technologies
CONSIGLIO NAZIONALE DELLE RICERCHE
Via Ampere, 56
20131 MILANO Italy

This person is:
- [ ] applicant only
- [X] applicant and inventor
- [ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality: SWITZERLAND

State *(that is, country)* of residence: ITALY

This person is applicant for the purposes of:
- [ ] all designated States
- [ ] all designated States except the United States of America
- [X] the United States of America only
- [ ] the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

RONCUCCI, Romeo DECEASED
Institute of Advanced Biomedical Technologies
CONSIGLIO NAZIONALE DELLE RICERCHE
Via Ampere, 56
20131 MILANO Italy

This person is:
- [ ] applicant only
- [X] applicant and inventor
- [ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality: ITALY

State *(that is, country)* of residence: ITALY

This person is applicant for the purposes of:
- [ ] all designated States
- [ ] all designated States except the United States of America
- [X] the United States of America only
- [ ] the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

CLERICI, Libero A.
Joint Research Center
Environment Institute
21027 ISPRA(VA)

This person is:
- [ ] applicant only
- [X] applicant and inventor
- [ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality: ITALY

State *(that is, country)* of residence: ITALY

This person is applicant for the purposes of:
- [ ] all designated States
- [ ] all designated States except the United States of America
- [X] the United States of America only
- [ ] the States indicated in the Supplemental Box

[X] Further applicants and/or (further) inventors are indicated on another continuation sheet.

Continuation of Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)

If none of the following sub-boxes is used, this sheet should not be included in the request.

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)* VEZZONI, Paolo Institute of Advanced Biomedical Technologies CONSIGLIO NAZIONALE DELLE RICERCHE Via Ampere, 56 20131 MILANO Italy	This person is: ☐ applicant only ☒ applicant and inventor ☐ inventor only *(If this check-box is marked, do not fill in below.)*
State *(that is, country)* of nationality: ITALY	State *(that is, country)* of residence: ITALY

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*	This person is: ☐ applicant only ☐ applicant and inventor ☐ inventor only *(If this check-box is marked, do not fill in below.)*
State *(that is, country)* of nationality:	State *(that is, country)* of residence:

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*	This person is: ☐ applicant only ☐ applicant and inventor ☐ inventor only *(If this check-box is marked, do not fill in below.)*
State *(that is, country)* of nationality:	State *(that is, country)* of residence:

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*	This person is: ☐ applicant only ☐ applicant and inventor ☐ inventor only *(If this check-box is marked, do not fill in below.)*
State *(that is, country)* of nationality:	State *(that is, country)* of residence:

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box

☐ Further applicants and/or (further) inventors are indicated on another continuation sheet.

Box No.V DESIGNATION OF STATES

The following designations are hereby made under Rule 4.9(a) *(mark the applicable check-boxes; at least one must be marked)*:

Regional Patent

- ☒ AP **ARIPO Patent:** GH Ghana, GM Gambia, KE Kenya, LS Lesotho, MW Malawi, SD Sudan, SZ Swaziland, UG Uganda, ZW Zimbabwe, and any other State which is a Contracting State of the Harare Protocol and of the PCT
- ☒ EA **Eurasian Patent:** AM Armenia, AZ Azerbaijan, BY Belarus, KG Kyrgyzstan, KZ Kazakhstan, MD Republic of Moldova, RU Russian Federation, TJ Tajikistan, TM Turkmenistan, and any other State which is a Contracting State of the Eurasian Patent Convention and of the PCT
- ☒ EP **European Patent:** AT Austria, BE Belgium, CH and LI Switzerland and Liechtenstein, CY Cyprus, DE Germany, DK Denmark, ES Spain, FI Finland, FR France, GB United Kingdom, GR Greece, IE Ireland, IT Italy, LU Luxembourg, MC Monaco, NL Netherlands, PT Portugal, SE Sweden, and any other State which is a Contracting State of the European Patent Convention and of the PCT
- ☒ OA **OAPI Patent:** BF Burkina Faso, BJ Benin, CF Central African Republic, CG Congo, CI Côte d'Ivoire, CM Cameroon, GA Gabon, GN Guinea, ML Mali, MR Mauritania, NE Niger, SN Senegal, TD Chad, TG Togo, and any other State which is a member State of OAPI and a Contracting State of the PCT *(if other kind of protection or treatment desired, specify on dotted line)* .....

National Patent *(if other kind of protection or treatment desired, specify on dotted line)*:

- ☒ AL Albania .....
- ☒ AM Armenia .....
- ☒ AT Austria .....
- ☒ AU Australia .....
- ☒ AZ Azerbaijan
- ☒ BA Bosnia and Herzegovina .....
- ☒ BB Barbados
- ☒ BG Bulgaria .....
- ☒ BR Brazil .....
- ☒ BY Belarus .....
- ☒ CA Canada
- ☒ CH and LI Switzerland and Liechtenstein
- ☒ CN China .....
- ☒ CU Cuba .....
- ☒ CZ Czech Republic .....
- ☒ DE Germany .....
- ☒ DK Denmark .....
- ☒ EE Estonia .....
- ☒ ES Spain .....
- ☒ FI Finland .....
- ☒ GB United Kingdom
- ☒ GE Georgia .....
- ☒ GH Ghana .....
- ☒ GM Gambia
- ☒ GW Guinea-Bissau .....
- ☒ HR Croatia
- ☒ HU Hungary .....
- ☒ ID Indonesia
- ☒ IL Israel .....
- ☒ IS Iceland
- ☒ JP Japan .....
- ☒ KE Kenya .....
- ☒ KG Kyrgyzstan .....
- ☒ KP Democratic People's Republic of Korea .....
- ☒ KR Republic of Korea .....
- ☒ KZ Kazakhstan .....
- ☒ LC Saint Lucia
- ☒ LK Sri Lanka
- ☒ LR Liberia
- ☒ LS Lesotho .....
- ☒ LT Lithuania
- ☒ LU Luxembourg
- ☒ LV Latvia
- ☒ MD Republic of Moldova .....
- ☒ MG Madagascar .....
- ☒ MK The former Yugoslav Republic of Macedonia .....
- ☒ MN Mongolia
- ☒ MW Malawi .....
- ☒ MX Mexico .....
- ☒ NO Norway
- ☒ NZ New Zealand .....
- ☒ PL Poland .....
- ☒ PT Portugal .....
- ☒ RO Romania
- ☒ RU Russian Federation .....
- ☒ SD Sudan
- ☒ SE Sweden
- ☒ SG Singapore
- ☒ SI Slovenia .....
- ☒ SK Slovakia .....
- ☒ SL Sierra Leone
- ☒ TJ Tajikistan .....
- ☒ TM Turkmenistan .....
- ☒ TR Turkey .....
- ☒ TT Trinidad and Tobago .....
- ☒ UA Ukraine .....
- ☒ UG Uganda .....
- ☒ US United States of America .....
- ☒ UZ Uzbekistan .....
- ☒ VN Viet Nam .....
- ☒ YU Yugoslavia .....
- ☒ ZW Zimbabwe .....

Check-boxes reserved for designating States (for the purposes of a national patent) which have become party to the PCT after issuance of this sheet:

- ☐ .....
- ☐ .....

Precautionary Designation Statement: In addition to the designations made above, the applicant also makes under Rule 4.9(b) all other designations which would be permitted under the PCT except any designation(s) indicated in the Supplemental Box as being excluded from the scope of this statement. The applicant declares that those additional designations are subject to confirmation and that any designation which is not confirmed before the expiration of 15 months from the priority date is to be regarded as withdrawn by the applicant at the expiration of that time limit. *(Confirmation of a designation consists of the filing of a notice specifying that designation and the payment of the designation and confirmation fees. Confirmation must reach the receiving Office within the 15-month time limit.)*

Box No. VI PRIORITY CLAIM ☐ Further priority claims are indicated in the Supplemental Box.

Filing date of earlier application (day/month/year)	Number of earlier application	Where earlier application is: national application: country	regional application:* regional Office	international application: receiving Office
item (1) 28 August 1997	MI97A001972	ITALY		
item (2)				
item (3)				

☐ The receiving Office is requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) *(only if the earlier application was filed with the Office which for the purposes of the present international application is the receiving Office)* identified above as item(s):

** Where the earlier application is an ARIPO application, it is mandatory to indicate in the Supplemental Box at least one country party to the Paris Convention for the Protection of Industrial Property for which that earlier application was filed (Rule 4.10(b)(ii)). See Supplemental Box.*

Box No. VII INTERNATIONAL SEARCHING AUTHORITY

Choice of International Searching Authority (ISA) *(if two or more International Searching Authorities are competent to carry out the international search, indicate the Authority chosen; the two-letter code may be used):* ISA /

Request to use results of earlier search; reference to that search *(if an earlier search has been carried out by or requested from the International Searching Authority):*
Date *(day/month/year)* Number Country *(or regional Office)*

Box No. VIII CHECK LIST; LANGUAGE OF FILING

This international application contains the following **number of sheets:**

request	:	05
description (excluding sequence listing part)	:	20
claims	:	03
abstract	:	01
drawings	:	04
sequence listing part of description	:	--
Total number of sheets	:	33

This international application is **accompanied by** the item(s) marked below:

1. ☐ fee calculation sheet
2. ☐ separate signed power of attorney
3. ☐ copy of general power of attorney; reference number, if any:
4. ☐ statement explaining lack of signature
5. ☐ priority document(s) identified in Box No. VI as item(s):
6. ☐ translation of international application into (language):
7. ☐ separate indications concerning deposited microorganism or other biological material
8. ☐ nucleotide and/or amino acid sequence listing in computer readable form
9. ☐ other *(specify)*:

Figure of the drawings which should accompany the abstract:

Language of filing of the international application: ITALIAN

Box No. IX SIGNATURE OF APPLICANT OR AGENT

Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the request).

F Minoja

Fabrizio MINOJA

Milan, 07 August 1998

For receiving Office use only

1. Date of actual receipt of the purported international application:
2. Drawings: ☐ received: ☐ not received:
3. Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application:
4. Date of timely receipt of the required corrections under PCT Article 11(2):
5. International Searching Authority (if two or more are competent): ISA /
6. ☐ Transmittal of search copy delayed until search fee is paid.

For International Bureau use only

Date of receipt of the record copy by the International Bureau:

PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

MINOJA, Fabrizio
BIANCHETTI BRACCO MINOJA S.R.L.
Via Rossini, 8
I-20122 Milano
ITALIE

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL PRELIMINARY EXAMINATION REPORT
(PCT Rule 71.1)

Date of mailing *(day/month/year)*	27.10.99
Applicant's or agent's file reference: SCB451PCT	**IMPORTANT NOTIFICATION**

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/IT98/00231	11/08/1998	28/08/1997

Applicant
CONSIGLIO NAZIONALE DELLE RICERCHE et al.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/	Authorized officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Vullo, C Tel.+49 89 2399-8061

PATENT COOPERATION TREATY

From the INTERNATIONAL SEARCHING AUTHORITY

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL SEARCH REPORT OR THE DECLARATION

(PCT Rule 44.1)

To:
BIANCHETTI BRACCO MINOJA S.R.L.
Attn. MINOJA, Fabrizio
Via Rossini, 8
I-20122 Milano
ITALY

Applicant's or agent's file reference SCB451PCT	Date of mailing *(day/month/year)* 21/01/1999 **FOR FURTHER ACTION** See paragraphs 1 and 4 below
International application No. PCT/IT 98/00231	International filing date *(day/month/year)* 11/08/1998
Applicant CONSIGLIO NAZIONALE DELLE RICERCHE et al.	

1. [X] The applicant is hereby notified that the International Search Report has been established and is transmitted herewith.

Filing of amendments and statement under Article 19
The applicant is entitled, if he so wishes, to amend the claims of the International Application (see Rule 46):

When? The time limit for filing such amendments is normally 2 months from the date of transmittal of the International Search Report; however, for more details, see the notes on the accompanying sheet.

Where? Directly to the International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland
Fascimile No.: (41-22) 740.14.35

For more detailed instructions, see the notes on the accompanying sheet.

2. [ ] The applicant is hereby notified that no International Search Report will be established and that the declaration under Article 17(2)(a) to that effect is transmitted herewith.

3. [ ] **With regard to the protest** against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:

[ ] the protest together with the decision thereon has been transmitted to the International Bureau together with the applicants's request to forward the texts of both the protest and the decision thereon to the designated Offices.

[ ] no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. **Further action(s):** The applicant is reminded of the following:

Shortly after **18 months** from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau as provided in Rules 90*bis*.1 and 90*bis*.3, respectively, before the completion of the technical preparations for international publication.

Within **19 months** from the priority date, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase until 30 months from the priority date (in some Offices even later).

Within **20 months** from the priority date, the applicant must perform the prescribed acts for entry into the national phase before all designated Offices which have not been elected in the demand or in a later election within 19 months from the priority date or could not be elected because they are not bound by Chapter II.

Name and mailing address of the International Searching Authority European Patent Office, P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Nancy Gamme

Form PCT/ISA/220 (January 1994)

NOTES TO FORM PCT/ISA/220

These Notes are intended to give the basic instructions concerning the filing of amendments under article 19. The Notes are based on the requirements of the Patent Cooperation Treaty, the Regulations and the Administrative Instructions under that Treaty. In case of discrepancy between these Notes and those requirements, the latter are applicable. For more detailed information, see also the PCT Applicant's Guide, a publication of WIPO.

In these Notes, "Article", "Rule", and "Section" refer to the provisions of the PCT, the PCT Regulations and the PCT Administrative Instructions respectively.

INSTRUCTIONS CONCERNING AMENDMENTS UNDER ARTICLE 19

The applicant has, after having received the international search report, one opportunity to amend the claims of the international application. It should however be emphasized that, since all parts of the international application (claims, description and drawings) may be amended during the international preliminary examination procedure, there is usually no need to file amendments of the claims under Article 19 except where, e.g. the applicant wants the latter to be published for the purposes of provisional protection or has another reason for amending the claims before international pbulication. Furthermore, it should be emphasized that provisional protection is available in some States only.

What parts of the international application may be amended?

Under Article 19, only the claims may be amended.

During the international phase, the claims may also be amended (or further amended) under Article 34 before the International Preliminary Examining Authority. The description and drawings may only be amended under Article 34 before the International Examining Authority.

Upon entry into the national phase, all parts of the international application may be amended under Article 28 or, where applicable, Article 41.

When?

Within 2 months from the date of transmittal of the international search report or 16 months from the priority date, whichever time limit expires later. It should be noted, however, that the amendments will be considered as having been received on time if they are received by the International Bureau after the expiration of the applicable time limit but before the completion of the technical preparations for international publication (Rule 46.1).

Where not to file the amendments?

The amendments may only be filed with the International Bureau and not with the receiving Office or the International Searching Authority (Rule 46.2).

Where a demand for international preliminary examination has been/is filed, see below.

How?

Either by cancelling one or more entire claims, by adding one or more new claims or by amending the text of one or more of the claims as filed.

A replacement sheet must be submitted for each sheet of the claims which, on account of an amendment or amendments, differs from the sheet originally filed.

All the claims appearing on a replacement sheet must be numbered in Arabic numerals. Where a claim is cancelled, no renumbering of the other claims is required. In all cases where claims are renumbered, they must be renumbered consecutively (Administrative Instructions, Section 205(b)).

The amendments must be made in the language in which the international application is to be published.

What documents must/may accompany the amendments?

Letter (Section 205(b)):

The amendments must be submitted with a letter.

The letter will not be published with the international application and the amended claims. It should not be confused with the "Statement under Article 19(1)" (see below, under "Statement under Article 19(1)").

The letter must be in English or French, at the choice of the applicant. However, if the language of the international application is English, the letter must be in English; if the language of the international application is French, the letter must be in French.

The letter must indicate the differences between the claims as filed and the claims as amended. It must, in particular, indicate, in connection with each claim appearing in the international application (it being understood that identical indications concerning several claims may be grouped),whether

(i) the claim is unchanged;

(ii) the claim is cancelled;

(iii) the claim is new;

(iv) the claim replaces one or more claims as filed;

(v) the claim is the result of the division of a claim as filed.

The following examples illustrate the manner in which amendments must be explained in the accompanying letter:

1. [Where originally there were 48 claims and after amendment of some claims there are 51]:
"Claims 1 to 29, 31, 32, 34, 35, 37 to 48 replaced by amended claims bearing the same numbers; claims 30, 33 and 36 unchanged; new claims 49 to 51 added."

2. [Where originally there were 15 claims and after amendment of all claims there are 11]:
"Claims 1 to 15 replaced by amended claims 1 to 11."

3. [Where originally there were 14 claims and the amendments consist in cancelling some claims and in adding new claims]:
"Claims 1 to 6 and 14 unchanged; claims 7 to 13 cancelled; new claims 15, 16 and 17 added." or
"Claims 7 to 13 cancelled; new claims 15, 16 and 17 added; all other claims unchanged."

4. [Where various kinds of amendments are made]:
"Claims 1-10 unchanged; claims 11 to 13, 18 and 19 cancelled; claims 14, 15 and 16 replaced by amended claim 14; claim 17 subdivided into amended claims 15, 16 and 17; new claims 20 and 21 added."

"Statement under article 19(1)" (Rule 46.4)

The amendments may be accompanied by a statement explaining the amendments and indicating any impact that such amendments might have on the description and the drawings (which cannot be amended under Article 19(1)).

The statement will be published with the international application and the amended claims.

It must be in the language in which the international appplication is to be published.

It must be brief, not exceeding 500 words if in English or if translated into English.

It should not be confused with and does not replace the letter indicating the differences between the claims as filed and as amended. It must be filed on a separate sheet and must be identified as such by a heading, preferably by using the words "Statement under Article 19(1)."

It may not contain any disparaging comments on the international search report or the relevance of citations contained in that report. Reference to citations, relevant to a given claim, contained in the international search report may be made only in connection with an amendment of that claim.

Consequence if a demand for international preliminary examination has already been filed

If, at the time of filing any amendments under Article 19, a demand for international preliminary examination has already been submitted, the applicant must preferably, at the same time of filing the amendments with the International Bureau, also file a copy of such amendments with the International Preliminary Examining Authority (see Rule 62.2(a), first sentence).

Consequence with regard to translation of the international application for entry into the national phase

The applicant's attention is drawn to the fact that, where upon entry into the national phase, a translation of the claims as amended under Article 19 may have to be furnished to the designated/elected Offices, instead of, or in addition to, the translation of the claims as filed.

For further details on the requirements of each designated/elected Office, see Volume II of the PCT Applicant's Guide.

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
SCB451PCT		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/IT98/00231	11/08/1998	28/08/1997
International Patent Classification (IPC) or national classification and IPC C12N15/00		
Applicant CONSIGLIO NAZIONALE DELLE RICERCHE et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 6 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 2 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☒ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
25/03/1999	27.10.99
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Paresce, D Telephone No. +49 89 2399 8995

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/IT98/00231

I. Basis of th report

1. This report has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.*):

Description, pages:

1-18	as originally filed				

Claims, No.:

1-12	as received on	05/10/1999	with letter of	30/09/1999	

Drawings, sheets:

1/4-4/4	as originally filed

2. The amendments have resulted in the cancellation of:

☐ the description, pages:
☐ the claims, Nos.:
☐ the drawings, sheets:

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-12
	No:	Claims	
Inventive step (IS)	Yes:	Claims	1-12
	No:	Claims	
Industrial applicability (IA)	Yes:	Claims	1-12
	No:	Claims	

2. Citations and explanations

see separate sheet

VI. Certain documents cited

1. Certain published documents (Rule 70.10)

and / or

2. Non-written disclosures (Rule 70.9)

see separate sheet

Re Item V

Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1) Reference is made to the following documents:

D1: GUVEN, K. ET AL.: 'Evaluation of a stress-inducible transgenic nematode strain for rapid aquatic toxicity testing' AQUATIC TOXICOLOGY, vol. 29, no. 1-2, June 1994, pages 119-137, XP002089378

D2: CA-A-2 088 379 (CANDIDO EDWARD P M ;STRINGHAM EVE G (CA); JONES DONALD (CA)) 30 July 1994

2) **Novelty: Article 33(2) PCT**

D1 discloses a transgenic strain of C. elegans which carries a construct comprising a hsp 70 gene promoter fused to a reporter gene sequence (a lacZ structural gene encoding β-galactosidase) (see abstract, and p.120). These transgenic C. elegans strains respond to stress by expressing the reporter gene and, therefore, are used to study the effects of heat shock or exposure to other environmental stress such as to various toxins. The transgenic worms are exposed to heat (p. 121, last paragraph- p.122, first paragraph) as well as to various toxicants such as heavy metals (Cd, Zn, Hg, Mn, Sn, Ag) (see Table 1). The effect of exposure to environmental stress is determined by measuring the induced β-galactosidase enzyme activity. D1 discloses that several heavy metals (Cd, Zn, Hg, Mn, Sn, Ag) cause dose-dependent transgene expression (abstract).

D2 discloses a transgenic strain of C. elegans which carries a construct comprising a hsp16 gene promoter fused to a reporter gene sequence (a lacZ structural gene encoding β-galactosidase) (see p. 2). These transgenic C. elegans strains are also used to study the effects of heat shock or exposure to other environmental stress such as to heavy metals. The methods for measuring the effects of various toxins on the transgenic worms are the same as those described in D1.

D1 and D2 do not disclose a transgenic mammal comprising cells containing a

construct of a hsp promoter fused to a reporter gene sequence. D1 and D2 do not disclose the use of the GH gene sequence as the reporter gene sequence, both documents only describe the use of the lacZ gene as the reporter gene. Therefore, the subject-matter of claims 1-12 has not been made available to the public by any of the available prior art documents and can therefore be regarded as novel.

3) **Inventive Step: Article 33(3) PCT**

The subject-matter of claims 1-12 cannot be derived from the available prior art in an obvious manner and therefore complies with the requirements of Article 33(3) PCT.

The closest prior art to evaluate the inventiveness of claims 1-12 is D1 or D2. D1 and D2 disclose stress-inducible transgenic nematode strains as well as the use of said transgenic animals to study the effects of exposure to environmental stress. The subject-matter of claims 1-12 differs from the teachings of D1 or D2 in that the present application discloses the use of transgenic mammals for toxicity studies and GH is used as a reporter gene rather than a lacZ reporter gene.

The problem to be solved by the present invention may be regarded as the provision of a system for toxicity studies in vivo, avoiding animal sacrifice and reducing the number of animals involved, but providing a system of detecting toxic effects in mammals. The solution to this problem proposed in claims 1-12 of the present application is considered as involving an inventive step (Article 33(3) PCT) for the following reasons:

The use of a GH reporter gene instead of a lacZ reporter gene allows for toxicity studies to be done in in vivo. The metabolic pathways in mammals (such as mice) are more closely related to those in humans than those of nematodes. Therefore, the use of transgenic mice rather than nematodes provides a system for toxicity studies that can provide useful data on the effects of toxins in humans.

Re Item VI

Certain documents cited

Certain published documents (Rule 70.10)

Application No Patent No	Publication date *(day/month/year)*	Filing date *(day/month/year)*	Priority date *(valid claim)* *(day/month/year)*
WO 98 28971 A	09.07.98	31.12.97	03.01.97

CLAIMS

1. A non-human transgenic animal which comprises cells containing a construct of a stress-sensitive regulatory sequence linked to a reporter-gene sequence.

2. A non-human transgenic animal according to claim 1, wherein said regulatory sequence is the heat shock protein (hsp) promoter.

3. A non-human transgenic animal according to claim 2, wherein said sequence is hsp70 gene promoter.

4. A non-human transgenic animal according to claims 1-3, wherein said reporter gene is the growth hormone (GH) gene.

5. A non-human transgenic animal according to any of the previous claims, which is a mammal.

6. A non-human transgenic animal according to claim 5, which is a rodent.

7. A non-human transgenic animal according to claim 6, which is a mouse.

8. A primary cell culture obtained from the transgenic animals of claims 1-7, wherein cells bear a construct of a stress-sensitive regulatory sequence linked to a reporter-gene sequence.

9. A primary cell culture according to claim 8, which is a fibroblast, hepatocyte, kidney, lung and bone marrow-cell culture.

10. A method for the study of chemical, physical and biological toxic agents which comprises:

a) exposing the transgenic animal of claims 1-7 to the toxic agent;

b) determining the effect through measurement of

Replaced by Article 34

the reporter-gene expression.

11. A method according to claim 10, wherein the same animal is used for repeated tests with the same or different toxic agent.

12. A method according to claims 10-11, for the study of toxicity kinetics of one or more toxic agents.

13. A method according to claims 10-12, for the study of heat stress.

14. A method according to claims 10-12, for the study of metal toxicity.

15. A method according to claim 14 for the study of toxicity of metals selected from the group consisting of Rb, Cu, Hg, As and Cd.

16. A method for the toxicity study of chemical, physical and biological agents, which comprises:

a) preparing a primary culture from the transgenic animal of claims 1-7, in which the cultured cells bear a construct of a stress-sensitive regulatory sequence linked to a reporter-gene sequence;

b) exposing the primary culture to the toxic agent;

c) determining the effect through the expression of the reporter gene in the culture medium.

17. A method according to claim 16, wherein fibroblast and hepatocyte primary cultures are used.

18. A method according to claims 16-17 for the study of metal toxicity.

19. A method according to claim 18, wherein metals are selected from the group consisting of Rb, Cr, Cu, Hg, As, and Cd.

20. The use of the transgenic animal of claim 1 for in vivo toxicity studies.

21. The use of a transgenic animal according to claim 19, wherein said animal is a mouse.

22. The use of primary cultures of cells from the transgenic animal of claim 1, for in vitro toxicity studies.

CLAIMS

1. A non-human transgenic mammal which comprises cells containing a construct of a heat shock protein (hsp) promoter linked to the growth hormone (GH) gene sequence.

2. A non-human transgenic mammal according to claim 1, wherein the heat shock protein promoter is hsp70 gene promoter.

3. A non-human transgenic mammal according to claim 1, which is a rodent.

4. A non-human transgenic mammal according to claim 3, which is a mouse.

5. A method for the study of chemical, physical and biological toxic agents which comprises:

a) exposing the transgenic mammal of claims 1-4 to the toxic agent;

b) determining the effect through measurement of the hematic concentration of the reporter-gene.

6. A method according to claim 5, wherein the same animal is used for repeated tests with the same or different toxic agent.

7. A method according to claims 5-6, for the study of toxicity kinetics of one or more toxic agents.

8. A method according to claims 5-6, for the study of heat stress.

9. A method according to claims 5-6, for the study of metal toxicity.

10. A method according to claim 9 for the study of toxicity of metals selected from the group consisting of Rb, Cu, Hg, As and Cd.

11. The use of the transgenic mammal of claim 1 for in vivo toxicity studies.

12. The use of a transgenic animal according to claim 11, wherein said animal is a mouse.

ATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION** see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
SCB451PCT		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/IT 98/ 00231	11/08/1998	28/08/1997
Applicant		
CONSIGLIO NAZIONALE DELLE RICERCHE et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 3 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. [ ] **Certain claims were found unsearchable** (see Box I).

2. [ ] **Unity of invention is lacking** (see Box II).

3. [ ] The international application contains disclosure of a **nucleotide and/or amino acid sequence listing** and the international search was carried out on the basis of the sequence listing
   - [ ] filed with the international application.
   - [ ] furnished by the applicant separately from the international application,
     - [ ] but not accompanied by a statement to the effect that it did not include matter going beyond the disclosure in the international application as filed.
   - [ ] Transcribed by this Authority

4. With regard to the **title,**
   - [ ] the text is approved as submitted by the applicant
   - [X] the text has been established by this Authority to read as follows:

   TRANSGENIC ANIMALS FOR THE STUDY OF BIOLOGICAL, PHYSICAL AND CHEMICAL TOXIC AGENTS

5. With regard to the **abstract,**
   - [X] the text is approved as submitted by the applicant
   - [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this International Search Report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is:

   Figure No. 1
   - [ ] as suggested by the applicant.
   - [X] because the applicant failed to suggest a figure.
   - [ ] because this figure better characterizes the invention.
   - [ ] None of the figures.

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 15/00, A01K 67/027, C12N 5/10

A1

(11) International Publication Number: WO 99/11772

(43) International Publication Date: 11 March 1999 (11.03.99)

(21) International Application Number: PCT/IT98/00231

(22) International Filing Date: 11 August 1998 (11.08.98)

(30) Priority Data:
MI97A001972 28 August 1997 (28.08.97) IT

(71) Applicant *(for all designated States except US)*: CONSIGLIO NAZIONALE DELLE RICERCHE [IT/IT]; Piazzale Aldo Moro, 7, I–00185 Roma (IT).

(72) Inventor: RONCUCCI, Romeo (deceased).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: SACCO, Maria, Grazia [IT/IT]; Institute of Advanced Biomedical Technologies, Consiglio Nazionale delle Ricerche, Via Ampere, 56, I–20131 Milano (IT). ZECCA, Luigi [IT/IT]; Institute of Advanced Biomedical Technologies, Consiglio Nazionale delle Ricerche, Via Ampere, 56, I–20131 Milano (IT). BROMLEY, Peter [CH/IT]; Institute of Advanced Biomedical Technologies, Consiglio Nazionale delle Ricerche, Via Ampere, 56, I–20131 Milano (IT). CLERICI, Libero, A. [IT/IT]; Joint Research Center, Environment Institute, I–21027 Ispra (IT). VEZZONI, Paolo [IT/IT]; Institute of Advanced Biomedical Technologies, Consiglio Nazionale delle Ricerche, Via Ampere, 56, I–20131 Milano (IT).

(74) Agent: MINOJA, Fabrizio; Bianchetti Bracco Minoja S.r.l., Via Rossini, 8, I–20122 Milano (IT).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published

With international search report.

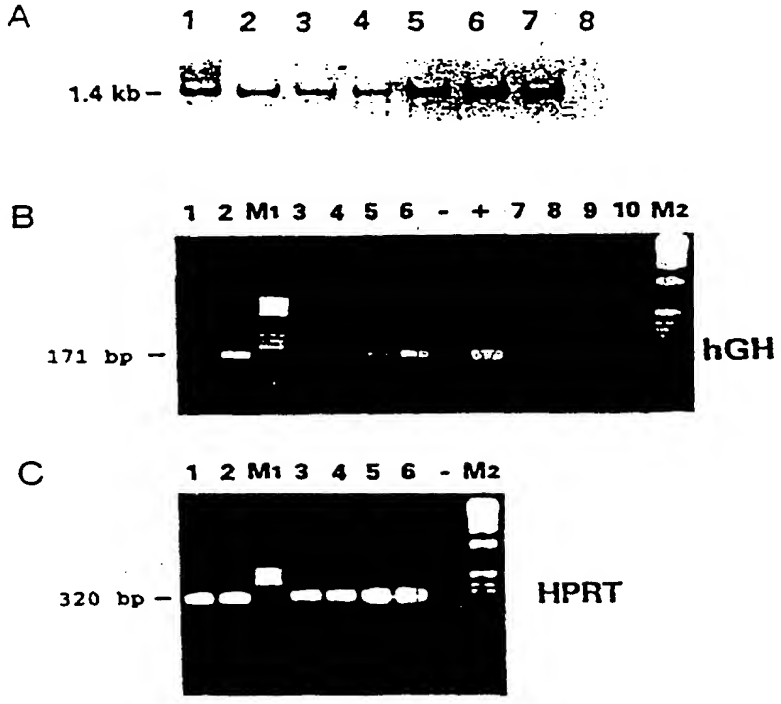
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

In English translation (filed in Italian).

(54) Title: TRANSGENIC ANIMALS FOR THE STUDY OF BIOLOGICAL, PHYSICAL AND CHEMICAL TOXIC AGENTS

(57) Abstract

The invention provides non–human transgenic animals bearing regulatory DNA sequences in some or all their cells, which are sensitive to biological, physical and chemical toxic agents. Such sequences are linked to sequences of reporter genes useful for toxicological studies.



FOR THE PURPOSES OF INFORMATION ONLY

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BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

TRANSGENIC ANIMALS FOR THE STUDY OF BIOLOGICAL, PHYSICAL AND CHEMICAL TOXIC AGENTS

The present invention provides transgenic animals for the study of biological, physical and chemical toxic agents.

At present, toxicity tests can be carried out both in vivo and in vitro.

The industrials, the public opinion and the scientific community are strongly interested in the abolition of toxicity tests made on animals and therefore in their replacement with in vitro tests.

This target, however, is quite unrealistic at the moment, since no in vitro tests which can replace in vivo tests are available, either now or in the near future.

It is well known, in fact, that the substances under in vivo investigation often undergo metabolic modifications, which might significantly alter their toxicity profile, to an extent which would be unpredictable in in vitro tests.

On the other hand, in vivo studies always involve animal suffering and sacrifice.

However, it is possible to conceive genetically-engineered animal models which may simplify the determination of the toxicity of various agents and reduce the number of animals involved.

Recently, the use of transgenic animals as models for pharmacological studies has been proposed.

For example, EP 0 169 672 B1 describes transgenic animals bearing oncogenes like c-myc, suitable for the

study of tumors associated to the expression of such oncogenes, or bearing the human growth hormone gene fused to a metallothionein promoter, whereby, said promoter being an inducible promoter, it is possible to study the effect of the expression, upon induction, of the associated gene on the whole organism (Palmiter et al. (1983) Science 222, 809).

WO 91/15579 describes a method for studying mutagenesis in transgenic animals bearing DNA sequences which can easily be extracted and analysed for mutations.

The present invention provides non-human transgenic animals useful for toxicity studies.

Such animals are characterised in that they have regulatory DNA sequences in some or all their cells, which are sensitive to biological, physical and chemical toxic agents, functionally linked to sequences of reporter genes, whereby the expression of the latter sequences is controlled or induced by said regulatory sequences.

Among the regulatory sequences, the stress-promoter sequences, like the heat shock protein (hsp) promoters, are preferred, but also cytochrome-promoters of the p450-superfamily, as well as those promoters of other genes, like p53 gene, activated by biological, chemical or physical stress, can be cited.

Among suitable reporter genes, the growth hormone gene, which has been used in the experiments described below, is preferred, but also chloramphenicol acetyl transferase (CAT), green fluorescence protein (GFP) and β-galactosidase (LacZ) genes can be suitably employed.

The transgenic animals of the invention can be used

in a method for studying the toxicity induced by various agents.

In theory, any animal normally suitable for a toxicity test can be used in the method of the invention. In practice, non-human mammals, particularly primates and rodents, are preferred.

Mice, in particular, are the most preferred.

Conventional methods can be used for the production of transgenic animals, including, for example, the microinjection of recombinant DNA into embryonal cells or into pronuclei of one-cell stage embryos, the zygote, embryo cell, somatic cell or animal tissue infection with a virus, in particular with a retrovirus, according to what described, for example, in Hogan et al., Cold Spring Harbor Laboratory Press, NY, 1986; Palmiter et al., Ann. Rev. Genet., 20: 465-499; 1986; Capecchi, Science, 244: 288-292, 1989.

The method for the in vivo assay of potential toxic compounds according to the present invention, comprises exposing the animal to a chemical or physical agent for a time sufficient to induce the effect, and simply measuring the reporter gene expression. When the reporter gene encodes a protein secreted in the bloodstream, for instance, its hematic concentration, as well as other chemical-clinical parameters associated with the effect caused by the activation of the stress promoter, could be detected.

According to the first aspect of the invention, a preferred embodiment is the production of transgenic mice in which a construct has been inserted, which comprises a hsp promoter fused to growth hormone (GH)

gene (transgene), said promoter being described in Dreano et al. (Biotechnology u6:953, 1988 and Gene 49:1-8, 1986) and in Fishbach et al. (Cell Biol. Toxicol. 9:177-188, 1993). The latter publication reports that the exposure to toxic metals of a stable fibroblast line, engineered with a construct containing the growth hormone gene under the control of hsp promoter, causes the secretion of the reporter gene in the medium.

According to the preferred embodiment of the invention, the injury caused by the toxic agent is determined as the increase of GH plasma concentration versus the control.

This model has resulted particularly efficient and sensitive, especially in relation with toxic metals, but it can suitably be used also for other classes of chemical toxic compounds, like endocrine disruptors, as well as for other physical or chemical agents, like radiations and electromagnetic fields.

The main advantages offered by the invention are: the possibility to diminish animal suffering, since only low amounts of the test substances are used, surely lower than the dosages which could induce animal suffering or death; the reduction of the number of animals used in toxicological tests; the provision of a model that is absolutely reliable for what concerns the metabolic modifications, which the toxic agents undergo in the organism, the interactions of toxic compounds with various organs and their final effects on cells, including the chronic effects. This model is particularly useful for test reiterations and allows to monitor the agent's effect during long-lasting

treatments using always the same animal, thus eliminating the variability of the individual response. Further, several compounds can be studied using the same animal. Finally, such transgenic models can be used also for in vivo studies of toxicity kinetics of toxic compounds.

The second aspect of the invention concerns the possibility to obtain primary cultures of cells from different tissues of the transgenic animal, in which a recombinant DNA construct is integrated as described above, whereby a cell- or tissue-specific toxicity study can be carried out and the intracellular biochemical effects connected to toxicity can be evaluated under controlled conditions and in more detail during different stages of animal growth.

In this case, the in vitro assay comprises preparing primary cultures in conditions variable depending on the cell type, exposing said cultures to the toxic agent and monitoring the activation of the stress promoter through detection of the protein encoded by the reporter gene.

Referring to the above described transgenic mice bearing the hsp/GH construct, an embodiment of the second aspect of the invention consists for example in preparing primary cultures of fibroblasts, kidney, lung or bone marrow cells, hepatocytes or other, in their simultaneous or separate treatment with one or more toxic agents, and in the determination of GH secretion in the medium.

If, using the above assay, a tissue or a cell-type resulted sensitive to the toxic agent, a deeper

biochemical analysis could be made in order to find which cellular pathways are particularly involved in the toxicity.

Thus, according to a further aspect, the invention provides a method to carry out in vitro toxicity tests on primary cultures of somatic cells derived from a transgenic animal.

BRIEF DECRIPTION OF THE FIGURES

Fig 1. Panel A: Southern blot analysis of transgenic heterozygous (lanes 1-4) and homozygous mice (lanes 5-7) and a non-transgenic control mouse (lane 8).

Panel B: RT-PCR with hGH specific primers of heat-shock activated liver cells from transgenic mice. Samples: RNA from cultured hepatocytes before (lane 1) and 30 min after (lane 2) heat shock in vitro; RNA from livers before (lane 3) and 30, 60, 90, minutes after heat shock (lanes 4-6). + and - represent the negative and positive controls respectively. Lanes 7 to 10 are the amplifications on non-retrotranscribed liver RNAs performed on the same samples as in lanes 3 to 6. M1: marker V, M2: 1 kb ladder.

Panel C: RT-PCR with HPRT specific primers performed on RNAs from the samples 1 to 6 as in panel B.

Fig. 2: Plasma levels of hGH (pg/ml) measured at different times in transgenic mice after thermal stress. Values represent the mean ± SE; the number of mice tested for each time period is indicated by the number above each bar.

Fig. 3: Mean hGH plasma levels (pg/ml) ± SE observed in transgenic mice injected i.p. with PBS and with various inorganic toxic compounds at the indicated

doses. Besides controls, are indicated: Rb: rubidium chloride; Hg: methylmercurium chloride; Cu: copper sulphate; Cd: cadmium chloride; As: sodium arsenite (2 doses)(below each bar is given the number of tested mice). The levels of significance are: $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.005$

Fig. 4: Mean ± SE of plasma hGH levels observed in transgenic mice subjected to two consecutive treatments, according to the following schema:

Group	First treatment (T_1)	Second treatment (T_2)	Time Interval (T_1-T_2)
As_1	As	As	10 days
As_2	Cd	As	2 months
As_3	Rb	As	2 months
Cu	Cu	Cu	2 months
Control	untretated	untreated	

The following examples better illustrate the invention:

EXAMPLE 1

Production and characterization of a transgenic mouse lineage

Transgenic mice were produced according to standard techniques (Hogan et al., "Manipulating the mouse embryo: a laboratory manual", Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986), by microinjecting 1-cell stage embryo pronuclei with a 1.4 kb EcoRI DNA fragment from p17hGH construct (described in Dreano et al., Biotechnology 6:953, 1988 and Gene

49:1-8, 1986), containing the human growth hormone cDNA as reporter gene, fused to the control region of the human Hsp70 promoter.

Mice were screened by Southern blot and/or PCR performed on tail DNA according to standard techniques. PCR was performed with the following primers: hGHL:GTGCAGTTCCTCAGGAGTGT; hGHR: CGAACTTGCTGTAGGTCTGC.

The amplification product was 171 bp long. Amplification conditions (35 cycles) were: 94°C for 20 sec, 58°C for 30 sec and 72°C for 20 sec. Heterozygous males and females were crossed and the homozygous progeny was identified by Southern blot, based on the intensity of the transgenic bands; their homozygosity was confirmed by checking the offspring when the homozygous male was mated to a non-transgenic partner. The mice used for the in vitro and in vivo experiments were always derived from a homozygous male bred with a non-transgenic CD-1 female.

Total RNA was extracted from different tissues (liver, spleen, lung, kidney, blood) of transgenic and control mice, according to standard techniques (Sambrook et al., "Molecular cloning: a laboratory manual", Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.). Southern and Northern blot were performed according to standard techniques.

In order to evaluate the basal value of non-induced expression of the transgene, mice were analysed with Northern blot and with RT-PCR.

No expression was detected in lung, kidney, spleen, liver and peripheral blood lymphocytes of non-treated animals or of animals not-exposed to heat shock. The hGH

level in non-treated mice (control) was generally under the test detection limits, and when it was determined, it never exceeded 10 pg/ml.

EXAMPLE 2

In vivo heat shock treatment.

Eight transgenic mice obtained according to example 1 and four non-transgenic control mice were subjected to in vivo heat shock at 44°C for 30 min. Six additional unexposed transgenic mice were tested. Aliquots of blood were taken before and 1, 3, 5, 7, and 24 hours after the heat shock.

In transgenic mice (Fig. 2) a specific increase of plasma hGH was detected with a peak three hour after treatment.

These results suggest that the integrated transgene does not affect in vivo the normal responsiveness of hsp promoter.

EXAMPLE 3

a) Inducibility of the hsp70/hGH transgene expression in vivo by sodium arsenite and methylmercurium chloride.

Male transgenic mice obtained as described in example 1 were weighed, anesthetized with ether and injected intraperitoneally (i.p.) with $NaAsO_2$ dissolved in PBS, at a final dose of 2.5 or 5 mg/kg, or with 3.5 mg/kg CH_3HgCl dissolved in PBS. Control transgenic mice were injected with the same volume of PBS (about 200 µl/mouse).

Blood samples were recovered before injection and 1, 3, 5, 7 and 24 hours after treatment.

hGH plasma levels at different times and doses are shown in Fig. 3.

Both the tested doses of $NaAsO_2$ gave a clear and statistically significant response.

The response peaked after 3-5 hours and turned to the basal level 24 hours after injection.

CH_3HgCl gave hGH peaks after 5-7 hours and baseline hGH values 24 hours after injection.

b) Following the same procedure as described in a), hGH inducibility was evaluated in mice treated with rubidium chloride (18.5 mg/kg, c), copper sulfate (9 mg/kg, d) and cadmium chloride (4.7 mg/kg, e).

Results are reported in Fig. 3.

EXAMPLE 4

Inducibility of the hsp70/hGH transgene expression in vivo by repeated injections of toxic compounds.

Initially, 13 mice were treated as follows:

5 mice with As, 3 mice with Cd, 2 mice with Rb, 3 mice with Cu. After a period of 10 days to 2 months, the former three groups of mice were re-inoculated with As, the latter with Cu.

Blood samples were taken before and 3-5 hours after injection, i.e. at the times of highest response.

As shown in Fig. 4, after the first administration of the compound, the mice showed a response comparable to that observed in groups of mice treated as in example 3.

When retested after 10-60 days, a similar hGH increase was observed.

EXAMPLE 5

Embryonic fibroblast primary cultures-in vitro toxicity tests.

Homozygous transgenic mice obtained as described in

example 1 were crossed with CD-1 females. After 14 days, embryonic fibroblasts (EMFIS) were recovered from the fetuses according to the technique described by Robertson E.J., IRL Press, Oxford, 77-88, 1987.

Cells were cultured in DMEM supplemented with 10% FCS and antibiotics (pen/strep), in an incubator (CO_2:5%, 100% humidity). Culture medium was replaced every second day with pre-warmed (37°C) fresh culture medium. The cells were expanded for two passages and then frozen at -80°C. For each experiment, cells were thawed, plated in 10 cm Petri dishes, left to grow and then re-seeded on 12 well plates until confluence.

To evaluate the toxic effect of the compounds, cells were treated by substituting the culture medium with fresh pre-warmed serum-free medium containing the toxic compounds at the chosen final dilutions. Cells were exposed to the toxic compound for either 5 or 24 hours and then the medium was replaced with fresh control medium for an additional 24 hours. At the end of the treatment, culture media were collected and assayed for hGH secretion by enzyme immunoassay.

Each treatment was performed in triplicate and the hGH determination was repeated twice for each plate. The results are expressed as pg of hGH/10^6 cells. The sensitivity of this method was approximately 2-4 pg/ml.

As shown in the table, calcium and rubidium, known for their lack of toxicity at the tested concentrations, do not provoke hGH release in the medium.

On the contrary, a significant release is induced after 24 hours of chrome exposure, while copper gives a low response after 24 hours at the highest

concentrations. On the contrary, mercurium does not induce hGH release from fibroblasts at each tested concentration.

Finally, arsenic and cadmium, as expected, showed clearly toxic.

EXAMPLE 6

Primary hepatocytes cultures-in vitro toxicity tests.

Transgenic male mice 8 weeks old were anesthetized and their livers were perfused as described in Clerici et al., Mut. Res., 227:47-51, 1989, in order to collect hepatocytes. Hepatocytes were then seeded on 24 well plates ($2x10^5$ cells/well) and cultured in William's E medium supplemented with antibiotics (pen/strep) and 10% FCS for 2 hours in order to allow them to attach to the bottom of the Petri dishes. The supernatant was then removed and the adherent cells were treated with the compounds dissolved in the medium.

To evaluate the toxic effect of the compounds, cells were treated by substituting the culture medium with fresh pre-warmed serum-free medium containing the toxic compounds at the chosen final dilutions.

As shown in the table, calcium and rubidium do not induce hGH release by mature hepatocytes.

Chrome treatment induces a high response after 24 hours, while copper treatment causes release either after 5 or 24 hours at each concentration.

Mercurium induces a response at concentrations higher than $5x10^{-5}$ M, while arsenic and cadmium show extremely toxic.

EXAMPLE 7

In vitro toxicity tests on kidney, lung and bone-marrow primary cultures.

Kidney and lung cells were recovered as described by Campbell, J. A. et al. ("Sister cromatid exchange analysis of mice following in vitro exposure to vinyl carbonate", In vitro Cell. Dev. Biol. 22: 443:448, 1986).

Briefly, kidneys were removed from the same animals subjected to liver perfusion, washed 3 times in PBS additioned with antibiotics and minced in 0.5 mm pieces with a sterile scalpel. After 1 hour of incubation in trypsin/collagenase (100U/ml) solution, the suspension was centrifuged twice for 5 min. at 50xg, plated in 100 mm Falcon dishes and cultured in McCoy's medium with 20% FCS, 2mM Glutamine and Pen/strep.

In order to collect lung cells, after liver perfusion the chest cavity was opened after liver perfusion to access the lungs. The trachea was cut with a scalpel and a 22-gauge catheter was inserted into the trachea to perfuse the lungs with trypsin/collagenase solution for 5 min. in order to help the disaggregation of this tissue. The cells were then trypsinized, seeded in 24 wells and left to grow until confluence in McCoy's medium with 20% FCS, 2mM Glutamine and antibiotics.

In order to prepare bone marrow primary cultures, bone marrow cells were flushexd from the cavity of femurs and tibias with a syringe containing the culture medium. Cells were plated in 12 well plates with McCoy's medium with 20% FCS, 2mM Glutamine and antibiotics, and left to grow until the stromal cells reached confluence.

To evaluate the toxic effect of the compounds, the

same procedure was followed as in the above examples 5 and 6.

Results are reported in the Table.

Table

(A) Determination of hGH (pg/10^6 cells) release and primary transgenic cultures viability after 5-hour treatment

Compounds	Primary lines	hGH release 10^{-5}M	$5x10^{-5}$M	10^{-4}M	$5x10^{-4}$M	Viabilty 10^{-5}M	$5x10^{-5}$M	10^{-4}M	$5x10^{-4}$M
$CaCl_2$	hepatocytes	nd	nd	nd	nd	+	+	+	+
RbCl		nd	nd	nd	nd	+	+	+	+
$CrCl_3$		/	nd	nd	nd	/	+	+	+
$CuSO_4$		/	nd	80	66	/	+	+	+
K_2Cr2O_7		nd	65	94	65	+/-	+/-	-	-
CH_3HgCl		nd	nd	nd	/	+/-	+/-	-	/
$CdCl_2$		309	452	57	14	+/-	+/-	-	-
$NaAsO_2$		100	224	nd	/	+	+/-	-	/
$CaCl_2$	Embryonic	/	nd	nd	nd	/	+	+	+
RbCl	fibroblast	/	nd	nd	nd	/	+	+	+
$CrCl_3$		/	nd	nd	nd	/	+	+	+
$CuSO_4$		/	nd	6	12	/	+	+	+
K_2Cr2O_7		9	nd	nd	nd	+/-	+/-	-	-
CH_3HgCl		/	nd	nd	nd	/	+/-	-	/
$CdCl_2$		250	85	45	nd	+/-	+/-	-	-
$NaAsO_2$		nd	113	19	nd	+	+/-	+/-	/

continues

$CaCl_2$	Kidney	/	nd	nd	nd	/	+	+	+
RbCl	cells	/	57	nd	nd	/	+	+	+
$CrCl_3$		/	nd	15	nd	/	+	+	+/-
$CuSO_4$		/	nd	nd	nd	/	+	+/-	+/-
$K_2Cr_2O_7$		nd	nd	nd	/	+/-	+/-	+/-	/
CH_3HgCl		10	nd	nd	/	+/-	+/-	+/-	/
$CdCl_2$		nd	nd	nd	/	+/-	-	-	/
$NaAsO_2$		22	17	28	/	+	+/-	-	/
$CaCl_2$	Lungs	/	nd	127	202	/	+	+	+
RbCl	cells	/	28	191	71	/	+	+	+
$CrCl_3$		/	92	122	166	/	+	+	+
$CuSO_4$		/	nd	nd	184	/	+/-	+/-	+/-
$K_2Cr_2O_7$		nd	nd	nd	/	+/-	-	-	/
CH_3HgCl		27	nd	nd	/	-	-	-	/
$CdCl_2$		nd	31	11	/	+/-	-	-	/
$NaAsO_2$		nd	37	249	/	+/-	+/-	-	/

hGH levels in untreated cells medium (controls) were not measurable after 5-24-hour incubation.

nd = undetectable; / = not determined; + = with 100% viability; with 30-70% viability;

- = 100% dead

(B) Determination of hGH (pg/10^6 cells) release and primary transgenic coltures after 24-hour treatment.

Compounds	Primary lines	hGH release 10^{-5}M	$5x10^{-5}$M	10^{-4}M	$5x10^{-4}$M	Vitality 10^{-5}M	$5x10^{-5}$M	10^{-4}M	$5x10^{-4}$M
$CaCl_2$	hepatocytes	nd	nd	nd	nd	+	+	+	+
RbCl		nd	nd	nd	nd	+	+	+	+
$CrCl_3$		/	36	20	nd	/	+	+	-
$CuSO_4$		/	12	61	100	/	+	+	+/-
$K_2Cr_2O_7$		nd	nd	nd	nd	-	-	-	-
CH_3HgCl		nd	63	103	/	+/-	-	-	/
$CdCl_2$		nd	nd	17	21	+/-	+/-	-	-
$NaAsO_2$		270	19	5	/	+	+/-	-	/
$CaCl_2$	Embryonic	/	nd	nd	nd	/	+	+	+
RbCl	fibroblast	/	nd	nd	nd	/	+	+	+
$CrCl_3$		/	8	10	6	/	+	+	+
$CuSO_4$		/	nd	10	47	/	+	+	+
$K_2Cr_2O_7$		nd	nd	nd	nd	+/-	-	-	-
CH_3HgCl		/	nd	nd	nd	/	-	-	-
$CdCl_2$		181	108	41	nd	-	-	-	-
$NaAsO_2$		19	380	37	4	+/-	+/-	-	-

continues

$CaCl_2$	Kidney	/	nd	nd	nd	/	+	+	+
RbCl	cells	/	nd	nd	nd	/	+	+	+
$CrCl_3$		/	nd	nd	nd	/	+	+	+/-
$CuSO_4$		/	nd	nd	450	/	+/-	+/-	-
$K_2Cr_2O_7$		nd	nd	nd	/	-	-	-	/
CH_3HgCl		nd	nd	nd	/	-	-	-	/
$CdCl_2$		nd	81	110	/	+/-	-	-	/
$NaAsO_2$		300	nd	40	/	+	+/-	-	/
$CaCl_2$	Lungs	/	nd	nd	nd	/	+	+	+/-
RbCl	cells	/	20	110	114	/	+	+	+/-
$CrCl_3$		/	200	199	35	/	+/-	+/-	+/-
$CuSO_4$		/	81	132	901	/	+/-	+/-	-
$K_2Cr_2O_7$		13	92	nd	/	-	-	-	/
CH_3HgCl		nd	164	nd	/	-	-	-	/
$CdCl_2$		64	196	415	/	+/-	-	-	/
$NaAsO_2$		20	55	nd	/	+/-	-	-	/
$CaCl_2$	Bone marrow	/	nd	51	nd	/	+	+	+/-
RbCl	cells	/	nd	20	128	/	+	+	+/-
$CrCl_3$		/	nd	21	21	/	+	+	+/-
$CuSO_4$		/	nd	127	145	/	+	+	-
$K_2Cr_2O_7$		nd	38	127	/	+/-	-	-	/
CH_3HgCl		nd	42	165	/	+/-	-	-	/
$CdCl_2$		nd	nd	nd	/	+/-	-	-	/
$NaAsO_2$		nd	nd	nd	/	+	+/-	+/-	/

hGH levels in untreated cells medium (controls) were not measurable after 24-hour incubation.
nd = undetectable; / = not determined; + = with 100% viability; with 30-70% viability;
- = 100% dead

CLAIMS

1. A non-human transgenic animal which comprises cells containing a construct of a stress-sensitive regulatory sequence linked to a reporter-gene sequence.

2. A non-human transgenic animal according to claim 1, wherein said regulatory sequence is the heat shock protein (hsp) promoter.

3. A non-human transgenic animal according to claim 2, wherein said sequence is hsp70 gene promoter.

4. A non-human transgenic animal according to claims 1-3, wherein said reporter gene is the growth hormone (GH) gene.

5. A non-human transgenic animal according to any of the previous claims, which is a mammal.

6. A non-human transgenic animal according to claim 5, which is a rodent.

7. A non-human transgenic animal according to claim 6, which is a mouse.

8. A primary cell culture obtained from the transgenic animals of claims 1-7, wherein cells bear a construct of a stress-sensitive regulatory sequence linked to a reporter-gene sequence.

9. A primary cell culture according to claim 8, which is a fibroblast, hepatocyte, kidney, lung and bone marrow-cell culture.

10. A method for the study of chemical, physical and biological toxic agents which comprises:

a) exposing the transgenic animal of claims 1-7 to the toxic agent;

b) determining the effect through measurement of

the reporter-gene expression.

11. A method according to claim 10, wherein the same animal is used for repeated tests with the same or different toxic agent.

12. A method according to claims 10-11, for the study of toxicity kinetics of one or more toxic agents.

13. A method according to claims 10-12, for the study of heat stress.

14. A method according to claims 10-12, for the study of metal toxicity.

15. A method according to claim 14 for the study of toxicity of metals selected from the group consisting of Rb, Cu, Hg, As and Cd.

16. A method for the toxicity study of chemical, physical and biological agents, which comprises:

a) preparing a primary culture from the transgenic animal of claims 1-7, in which the cultured cells bear a construct of a stress-sensitive regulatory sequence linked to a reporter-gene sequence;

b) exposing the primary culture to the toxic agent;

c) determining the effect through the expression of the reporter gene in the culture medium.

17. A method according to claim 16, wherein fibroblast and hepatocyte primary cultures are used.

18. A method according to claims 16-17 for the study of metal toxicity.

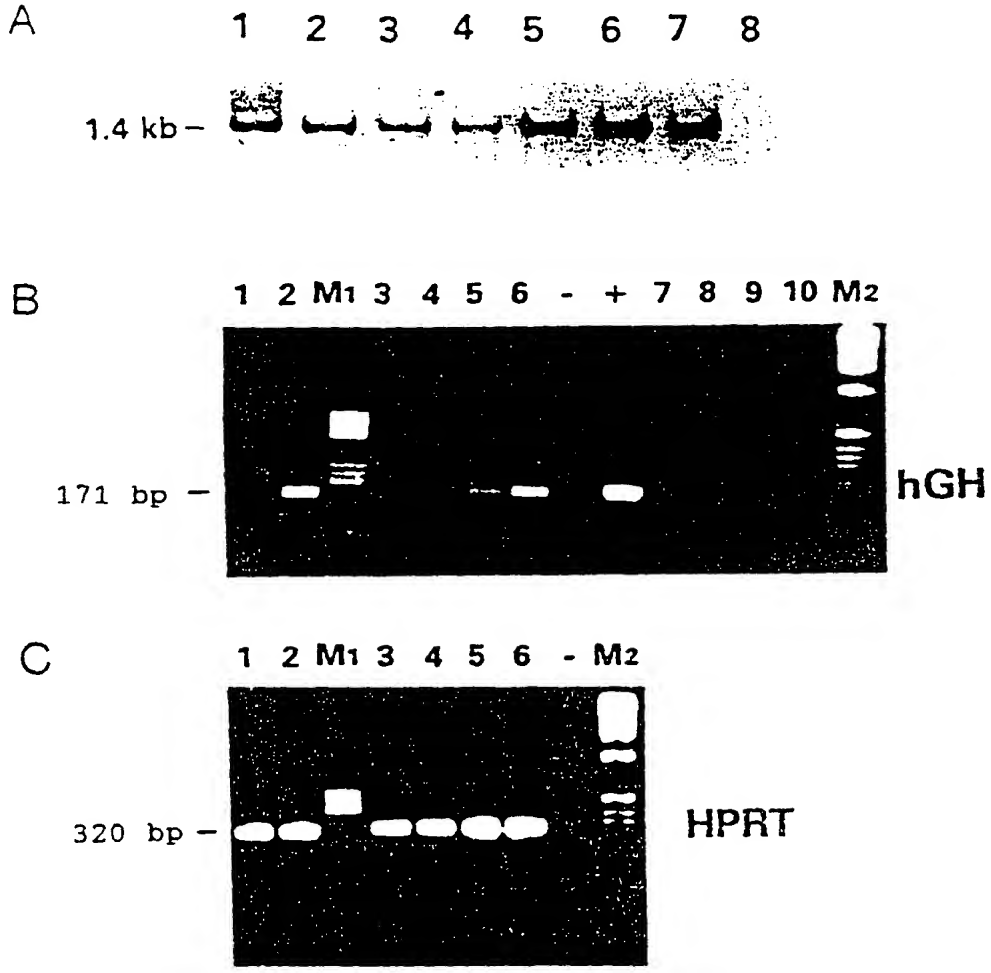
19. A method according to claim 18, wherein metals are selected from the group consisting of Rb, Cr, Cu, Hg, As, and Cd.

20. The use of the transgenic animal of claim 1 for in vivo toxicity studies.

21. The use of a transgenic animal according to claim 19, wherein said animal is a mouse.

22. The use of primary cultures of cells from the transgenic animal of claim 1, for in vitro toxicity studies.

FIGURE 1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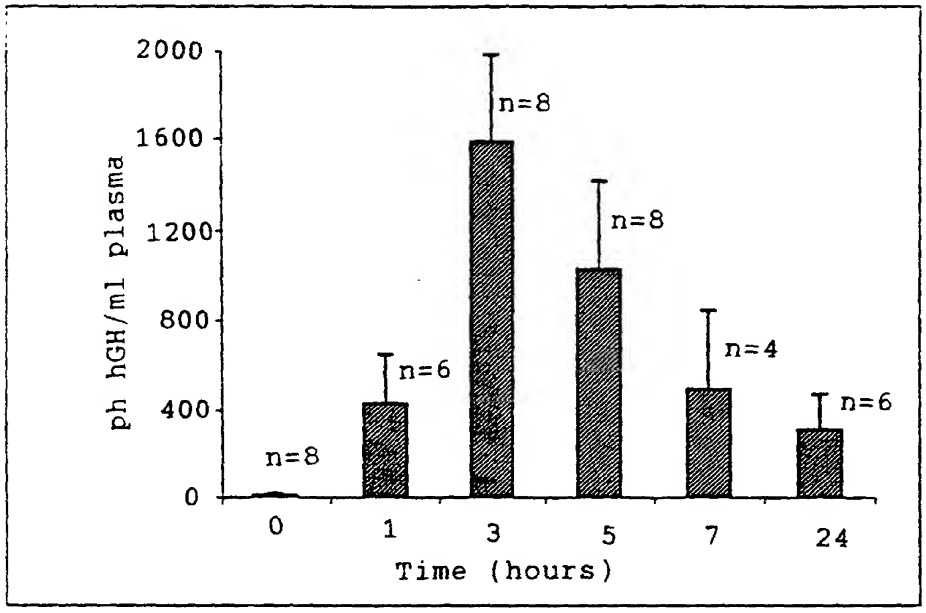
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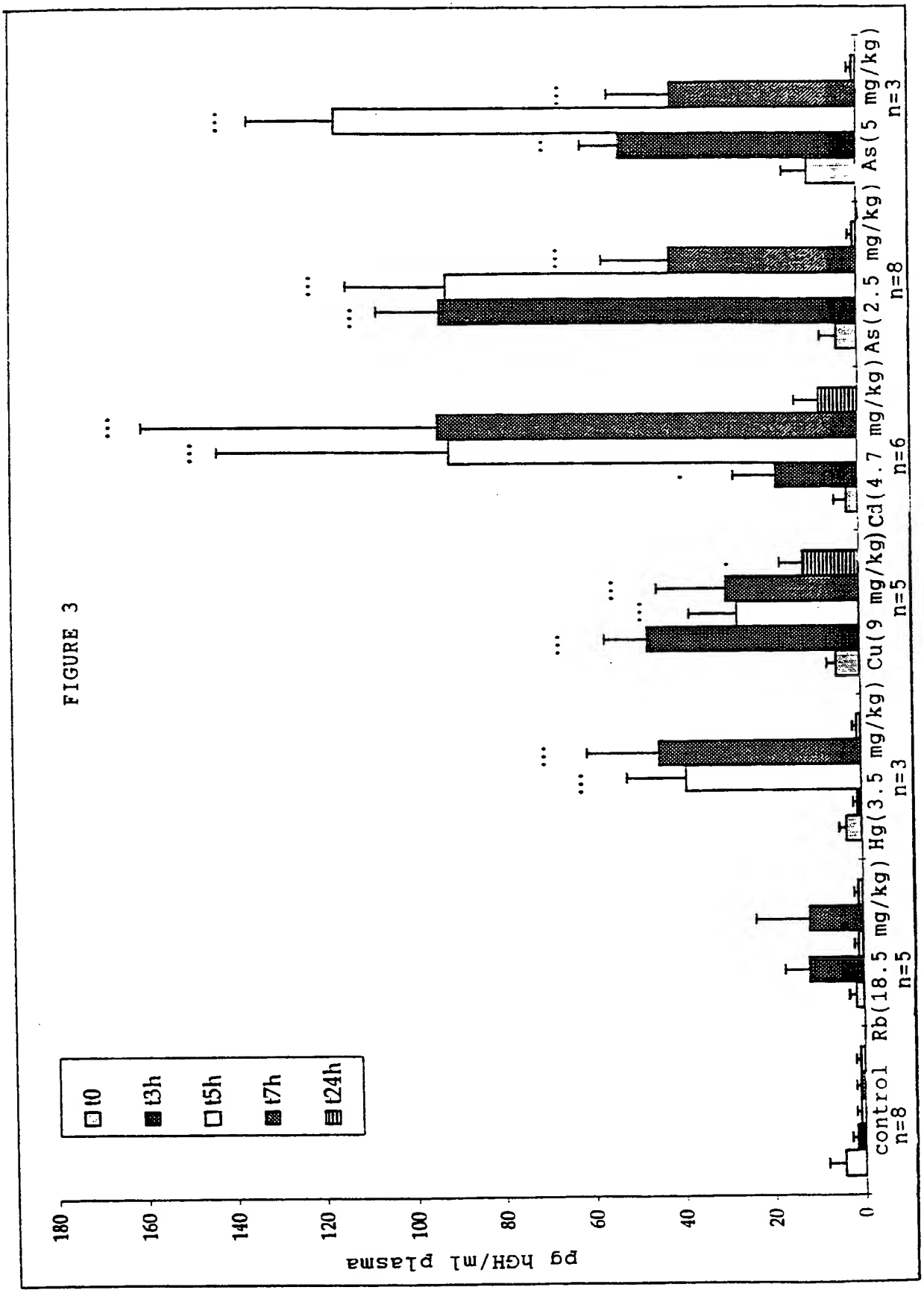
5
7
24
Time (hours)

FIGURE 2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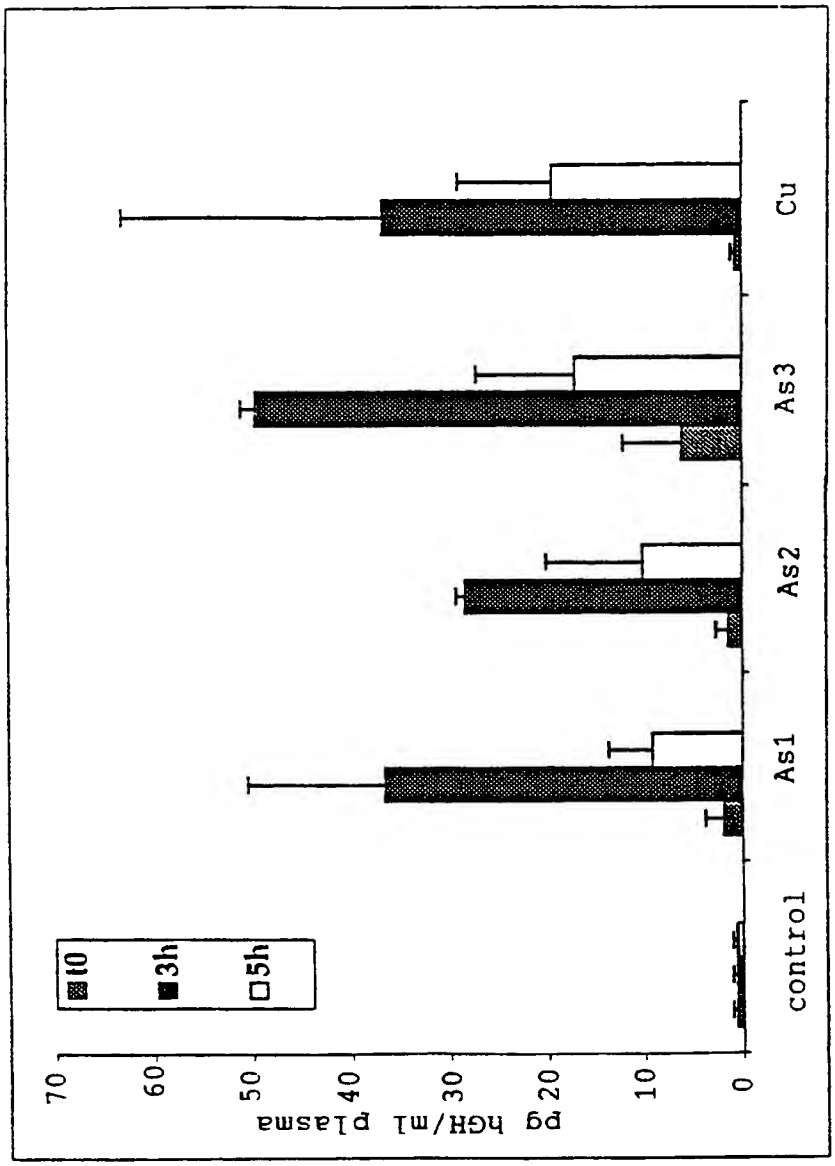
t24h
control n=8
Rb(18.5 mg/kg) n=5
Hg(3.5 mg/kg) n=3
Cu(9 mg/kg) n=5
Cd(4.7 mg/kg) n=6
As(2.5 mg/kg) n=8
As(5 mg/kg) n=3

FIGURE 3

FIGURE 4


10
3h
5h
pg hGH/ml plasma
0
10
20
30
40
50
60
70
control
As1
As2
As3
Cu

INTERNATIONAL SEARCH REPORT

International Application No
PCT/IT 98/00231

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/00 A01K67/027 C12N5/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A01K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GUVEN, K. ET AL.: "Evaluation of a stress-inducible transgenic nematode strain for rapid aquatic toxicity testing" AQUATIC TOXICOLOGY, vol. 29, no. 1-2, June 1994, pages 119-137, XP002089378	1-3, 10-15,20
Y	see the whole document	1-22
X	CA 2 088 379 A (CANDIDO EDWARD P M ;STRINGHAM EVE G (CA); JONES DONALD (CA)) 30 July 1994 see the whole document	1-3, 10-12,20
Y	EP 0 336 523 A (INTRACEL CORP) 11 October 1989 see the whole document	1-22

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	*Date of mailing of the international search report*
7 January 1999	21/01/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Chambonnet, F

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	EP 0 263 908 A (BATTELLE MEMORIAL INSTITUTE) 20 April 1988 see the whole document	1-22
P,X	WO 98 28971 A (LINK CHRISTOPHER ;UNIV TECHNOLOGY CORP (US)) 9 July 1998 see the whole document	1-3, 10-12,20
P,X	SACCO, M.G. ET AL.: "A transgenic mouse model for the detection of cellular stress induced by toxic inorganic compounds" NATURE BIOTECHNOLOGY., vol. 15, no. 13, December 1997, pages 1392-1397, XP002089379 UBLISHING US see the whole document	1-22

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/IT 98/00231

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